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Growth of *Streptomyces aureofaciens* in Continuous Culture

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Received for publication August 20, 1960

The technique of continuous cultivation of microorganisms is in many respects superior to the traditional cultivation methods, and has therefore been used for many purposes. One of its great advantages over classical methods is the possibility of holding a growing population at constant size over long periods of time. Another advantage lies in the fact that the concentration of all chemical substances in the cultivation vessel remains constant.

The theoretical basis for the continuous cultivation has been elaborated only recently, simultaneously by Monod (1950), and Novick and Szilard (1950), although the technique itself had been used before the solution of some theoretical and practical problems (Rogers and Whittier, 1930; Cleary, Beard, and Clifton, 1935; Málek, 1943). Since that time, the theory has been further developed by a number of authors in some special questions. Most of the published work is concerned with the growth of bacteria in continuous culture, but the growth of yeasts, actinomycetes, algae, flagellata, and animal cells has been studied as well. Although lately a number of papers concerned with the continuous cultivation of actinomycetes have been published (Brown, 1959; Bartlett and Gerhardt, 1959; Sikyta, Doskočil, and Kašparová, 1959), it seems that an exact measuring of the growth of actinomycetes under various steady states has not so far been undertaken.

It has been the aim of the present work to study the behaviour of the actinomycete *Streptomyces aureofaciens* as a representative of filamentous organisms, under exactly defined conditions of continuous growth, while using two nutrients as limiting factors, and to find out to what extent this behaviour is in accordance with the theory of continuous cultivation.

Materials and Methods

Organism. *S. aureofaciens* strain BMK from the collection of the Antibiotics Research Institute in Roztoky near Prague was used.

Medium. The composition of the synthetic fermentation medium was as follows: sucrose, 2 per cent; ammonium sulphate, 0.5 per cent; potassium phosphate monobasic, 0.68 per cent; sodium hydroxide, 0.125 per cent; magnesium sulphate heptahydrate, 0.05 per cent; sodium chloride, 0.2 per cent. The individual components of the medium were dissolved in distilled water and the medium was sterilized in a sterilization tank with mixing at 120 C for 20 min. After sterilization, the pH was 6.8 to 7.0. In this medium either sucrose (0.2 or 0.4 per cent) or ammonium sulphate (0.05 or 0.1 per cent) were used as growth limiting factors, the other components being present in excess.

Continuous culture apparatus. The equipment for the continuous cultivation consisted of (a) a tank of 100-L working capacity in which the nutrient medium was prepared and sterilized; (b) a smaller tank of 20-L working capacity which was used for storing the nutrient medium while another batch of the medium was being prepared in the sterilization tank; and (c) a fermentor which was of a design described in detail by Sikyta *et al.* (1958). This fermentor has an internal diameter of 24 cm. and a height of 49 cm; the working capacity is 10 L. It is equipped with an open-turbine-type agitator (six flat blades with a diameter of 12 cm), a sparger and four baffles. The agitating velocity was 400 rpm, the air flow 0.5 volume per volume of culture per min. The oxygen transfer rate, determined by the method of Cooper, Fernstrom, and Miller (1944) was 1.5 ml O_2 per ml per hr. The feed-rate of the medium into the fermentor was adjusted by means of a peristaltic pump and the level of the culture fluid in the vessel was maintained constant by an overflow tube. The fermented medium was collected in a collector. In all experiments described in this paper, the temperature in the culture vessel was 28 C.

The sterile synthetic medium in the fermentor was invariably inoculated with 50 ml of a 24-hr-old inoculum, grown in flasks on a reciprocal shaking machine. The synthetic medium was used as inoculating medium in the flasks with the addition of 0.1 per cent corn steep liquor (solids); it had been demonstrated that experiments run in tanks inoculated with an inoculum grown in a synthetic medium without corn steep did not furnish reproducible results.

Analytical methods. Samples were taken every 3 hr and tested for pH value and dry weight of the mycelium; samples were tested every 6 hr for ammonia-nitrogen content and sucrose concentration, and the chlortetracycline content was assayed biologically according to Hess (1955); the purity of the microbial species was examined in a sample taken every 12 hr. In

[1] All symbols used in this work were taken from the paper of Herbert Elsworth, and Telling (1956).

some cases, the amino nitrogen content (Schroeder, Kay, and Mills, 1950) of the medium and the nitrogen in the dry weight of the mycelium was determined.

For analytical assays 100 ml of the sample were withdrawn to flasks containing 1 ml of a 50 per cent solution of mercuric chloride.

Results

Growth in batch culture. To insure that the limiting substrate used was in fact the only limiting substrate, experiments were run in fermentors using a synthetic medium with the following concentrations of sucrose: 0, 0.2, and 0.4 per cent, and the following concentrations of ammonium sulphate: 0, 0.05, and 0.1 per cent. When zero concentrations of the limiting substrates were used, no growth occurred; with the remaining concentrations the termination of the logarithmic phase (in fact, practically the termination of growth altogether, for the logarithmic phases ended rather abruptly) corresponded well with the complete depletion of the limiting factor.

The values of the dry weight of mycelium, obtained at 3-hr intervals, were used for determining the maximal growth rate (μ_m).[1] For the logarithmic phases of the growth curves (natural logarithms of the dry weight of mycelium plotted against time) which took in individual experiments 15 to 21 hr, the slopes of the straight lines were determined by calculating their regression coefficients; this was done for both concentrations of the two limiting factors.

From the values obtained, the average value of the slope, 0.18, was calculated, *i.e.*, the average value of the maximal, attainable growth rate of the strain *S. aureofaciens* in the synthetic medium used. This value determines at the same time the maximal, theoretical dilution rate, which can be used for the cultivation of this microorganism without having it washed out from cultivation vessel.

Growth in continuous culture. The experiments were performed by allowing the cultivation (after inoculating the nutrient broth in the tank) to proceed batchwise until a concentration of the organisms reached 80 to 90 per cent of the expected value. This concentration usually takes place between 18 to 24 hr. Using lower dilution rates (lower than 0.08 hr^{-1}) the steady state became settled after exchanging approximately one volume of the nutrient broth; whereas, using dilution rates over 0.08 hr^{-1}, the steady-state was achieved after exchanging approximately two volumes of the nutrient broth in the fermentor. Therefore, measurements were performed only after exchanging the respective volumes. We proceeded in this way not only when starting the first run of an experiment, but also when changing one steady state for another, *i.e.*, when alternating between a higher and a lower dilution rate. The actual measuring of the values at a given dilution rate were performed for a period of 27 to 65 hr, *after* which the dilution rate was changed. This was continued until an increase occurred in the dry weight of the mycelium, due to growth of the microorganisms on the walls of the vessel, or until contamination by another microorganism or some mechanical defect made it necessary to interrupt the experiment.

The dry weight of mycelium remained at a given dilution rate (and within the margin of experimental error) constant for the entire duration of the measuring. As growth limiting factors, sucrose in 0.2 and 0.4 per cent concentration and ammonium sulphate in 0.05 and 0.1 per cent concentration were used.

Sucrose limitation. It can be seen from figures 1 and 2 that the values of the dry weight of mycelium remain constant within a certain range of dilution rate ($D = 0.052$ to 0.17 hr^{-1}). When the D_M value is exceeded, a decrease of the dry weight of mycelium takes place. Complete washing out of the organisms occurs at a dilution rate about 0.2 hr^{-1}, this value, obtained by extrapolation, is denoted D_C in the figure. A drop in the dry weight of mycelium was observed even at dilution rates lower than 0.052 hr^{-1}. The nitrogen content in the dry weight of the mycelium is approximately constant at all dilution rates examined, about 11.5 per cent (figure 3). The output of the dry weight of mycelium increases with increasing dilution rate up to D_M, after which it drops abruptly. In the range of dilution rates, 0.02 to 0.052 hr^{-1}, the output is smaller in accordance with the lower weight of dry mycelium. The yield constant has a constant value 0.43 in the range of dilution rates, 0.052 to 0.17 hr^{-1}; at dilution rates below 0.052 hr^{-1} and above 0.17 hr^{-1}, it decreases (figure 4). The substrate concentration is practically nil up to the dilution rate 0.17

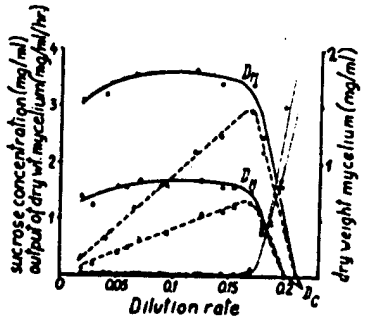

Figure 1. Steady-state relationships in continuous culture of *Streptomyces aureofaciens* using sucrose as the limiting factor in the inflowing medium. Media: 0.4 per cent sucrose and (●) dry weight mycelium, (○) sucrose concentration, (◎) output of dry weight mycelium; 0.2 per cent sucrose and (▲) dry weight mycelium, (△) sucrose concentration, (▲) output of dry weight mycelium.

hr $^{-1}$, whereupon the substrate begins to appear in the medium.

Very interesting is the correlation between the production of the antibiotic and the dilution rate (figure 2). At low dilution rates (lower than about 0.05 hr $^{-1}$) no antibiotic is produced. At higher dilution rates (over 0.052 hr $^{-1}$ up to D_{max}, the concentration of the antibiotic in the broth is held at a constant value. Because the dry weight of mycelium (in the said range of dilution rate) remains constant as well, the ratio chlortetracycline to 1 g dry weight of mycelium is also constant. At higher values of dilution rates (when $D = 0.17$ hr $^{-1}$ is exceeded) the content of the antibiotic in the nutrient broth varies considerably and is therefore not listed in the figures.

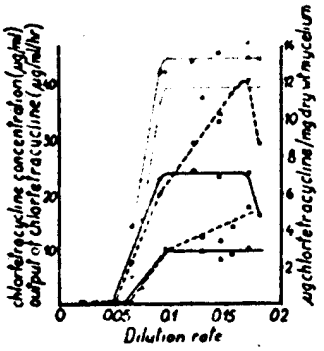

Figure 2. Steady-state relationships in continuous culture of *Streptomyces aureofaciens* using sucrose as the limiting factor in the following medium: Means: 0.4 per cent sucrose and ● chlortetracycline concentration, ○ chlortetracycline dry weight mycelium, ○ output of chlortetracycline; 0.2 per cent sucrose and ▲ chlortetracycline concentration, (△) chlortetracycline dry weight mycelium, (△) output of chlortetracycline.

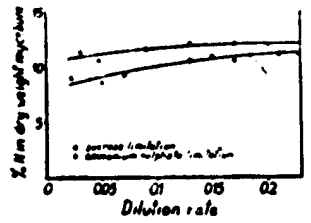

Figure 3. The influence of the type of limiting factor used on the N content in the dry weight of mycelium at different dilution rates.

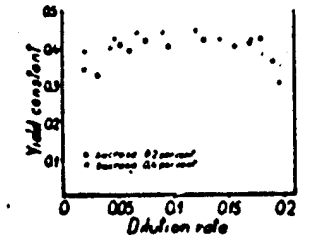

Figure 4. Relationship between the yield constant values and dilution rate in sucrose limitation.

Similar to the production of the antibiotic, the production of yellow pigments is also dependent on the dilution rate. Exact determinations of their concentration, *e.g.* colorimetric, would be rather difficult: when attempts were made to isolate them, they were shown to be composed of a complex of at least 6 compounds of different colors (unpublished experiments), therefore, these pigments were merely estimated by visual observation. At those dilution rates where no antibiotic is produced, pigments are not excreted to the medium, and the medium is whitish. At dilution rates above 0.05 hr $^{-1}$, excretion of pigments to the medium starts simultaneously with the production of the antibiotic, and the medium becomes yellow. Strong pigmentation of the medium and mycelium occurs at dilution rates higher than 0.08 hr $^{-1}$. It follows from these observations that there is a close relationship between the production of the antibiotic and that of the pigments. A nonpigmented mycelium, producing no antibiotic, appears at dilution rates lower than 0.05 hr $^{-1}$ after 40 to 80 hr of continuous growth. The time necessary for this depends on the dilution rate in such a way, *e.g.*, with a diminishing value of the dilution rate that the time necessary for the disappearance of the antibiotic and pigments at both concentrations of the limiting factors grows shorter.

When the dilution rate is altered repeatedly during one single run, the nonpigmenting mycelium grown at a dilution rate lower than 0.05 hr $^{-1}$ is found to start producing the pigments on increasing the dilution rate over 0.05 hr $^{-1}$, and the production of the pigments ceases again upon decreasing the dilution rate below 0.05 hr $^{-1}$; for that reason the process may be assumed to be reversible. Simultaneously with these changes in pigmentation the concentration of the antibiotic changes correspondingly.

These observations apply to both concentrations of the limiting substrate.

Ammonium sulphate limitation. Figures 5 and 6 summarize the results obtained during continuous growth of a culture of *S. aureofaciens* on a synthetic medium with the limiting concentrations of 0.05 and 0.1 per cent of ammonium sulphate. The relationship between the values of the dry weight of mycelium and the dilution rate (figure 5) is the same as in similar experiments with sucrose limitation. At very low dilution rates, the values of the dry weight of mycelium decrease

for both concentrations of ammonium sulphate used. The shape of the output curve of the dry weight of mycelium is analogous to sucrose limitation. The nitrogen content in the dry weight of mycelium (figure 5) decreases at lower dilution rates from an average value of 11 per cent to as low as 8 to 9 per cent. This decrease, and also a generally lower nitrogen content in the dry weight of mycelium grown at all dilution rates used, differentiates the ammonium sulphate limitation from sucrose limitation. The substrate first appears at the dilution rate 0.16 hr $^{-1}$, *i.e.*, somewhat sooner than is the case with sucrose being used as the limiting factor.

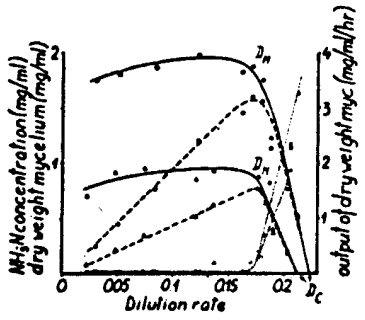

Figure 5. Steady-state relationships in continuous culture of *Streptomyces aureofaciens* using $(NH_4)_2SO_4$ as the limiting factor in the inflowing medium. Media: 0.1 per cent $(NH_4)_2SO_4$ and (●) dry weight mycelium, (○) NH_4-N concentration, (⊙) output of dry weight mycelium; 0.05 per cent $(NH_4)_2SO_4$ and (▲) dry weight mycelium, (△) NH_4-N concentration, (▲) output of dry weight mycelium.

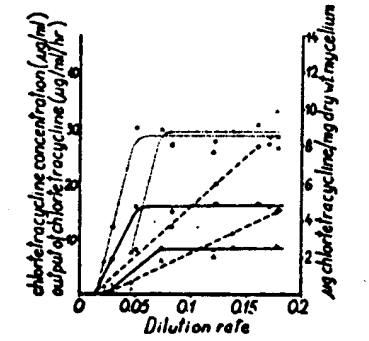

Figure 6. Steady state relationships in continuous culture of *Streptomyces aureofaciens* using $(NH_4)_2SO_4$ as the limiting factor in the inflowing medium. Media: 0.1 per cent $(NH_4)_2SO_4$ and (●) chlortetracycline concentration; (○) chlortetracycline dry weight mycelium, (⊙) output of chlortetracycline; 0.05 per cent $(NH_4)_2SO_4$ and (▲) chlortetracycline concentration, (△) chlortetracycline/dry weight mycelium, (▲) output of chlortetracycline.

The dry weight of mycelium was proportional to the concentration of the limiting substrate in the feed.

Principally, the ammonium sulphate limitation differs from sucrose limitation in antibiotic and pigment formation. With nitrogen limitation, both the antibiotic and the pigments are produced even at very low dilution rates (figure 6). It is possible that at still lower dilution rates (slower than 0.02 hr $^{-1}$, which was the lowest dilution rate used) the antibiotic and the pigments would disappear, but this could not be verified for technical reasons. At any rate, the loss of the ability to produce the antibiotic and the pigments is, with nitrogen limitation, shifted to extremely low values of dilution rates. In trial experiments run at the dilution rate 0.022 hr $^{-1}$, the production of the antibiotic and the pigments did not stop even after 150 hr of growth. With nitrogen limitation, the amount of the antibiotic produced is somewhat less than with sucrose limitation, although the values of the dry weight of mycelium are higher with nitrogen limitation than in corresponding runs with sucrose limitation. This is why the total output of the antibiotic and the amount of antibiotic per unit of the dry weight of mycelium is lower, as compared with sucrose limitation.

Discussion

Comparison of Behaviour of Filamentous with Nonfilamentous Organisms in Continuous Culture

The theory of continuous cultivation, elaborated by Monod (1950) and Novick and Szilard (1950), was extensively verified, especially by Herbert, Elsworth, and Telling (1956), for nonfilamentous organisms. As it has not been so far ascertained exactly to what extent the behaviour of filamentous organisms in continuous culture is in agreement with the laws derived for nonfilamentous organisms, we tried to verify this theory for filamentous organisms.

From the curves in figures 1, 4, and 5, it can be seen that *S. aureofaciens* behaves in continuous culture in accordance with assumptions derived by Monod, except for the decrease of dry weight of mycelium at low dilution rates and at those which are higher than a maximal growth rate. These variations are of the same character as those of nonfilamentous microorganisms and the possible cause shall be discussed later. According to the theory of the continuous cultivation, the behaviour of organisms in continuous culture with substrate limitation is characterized by three constants: the maximal growth rate (μ_m), the yield constant (Y), and the saturation constant (K_s).

Maximal growth rate (μ_m). The maximal growth rate of *S. aureofaciens* in batch cultivation is about 0.18 hr $^{-1}$, irrespective of the type or concentration of the limiting factor used. As compared with the maximal growth rate of bacteria and yeasts, this value is about five times

smaller (Monod, 1950, Herbert *et al.*, 1956; Holme, 1957). It is well known that the value of the maximal growth rate is dependent on the composition of the medium, from that it may appear that the low maximal growth rate is the result of the use of a synthetic medium. In our case, however, this is not so, for even a natural medium containing corn steep liquor failed to yield a higher maximal growth rate (unpublished data). As can be seen from figures 1 and 5, the critical dilution rate, D_c (at this rate the microorganisms would be, under ideal conditions, completely washed out from the cultivation vessel), is invariably higher than the maximal growth rate determined in batch cultivation (figure 1). This fact, often quoted by other authors for nonfilamentous organisms, is largely explained by the growth of microorganisms on the walls of the fermentation vessel or by imperfect mixing (Herbert *et al.*, 1956). Pirt (1957) states that this phenomenon is caused by a better adaptation of microorganisms to the cultivation conditions during a longer continuous cultivation.

Yield constant (Y). It proved impossible to determine reliably the yield constant from batch cultivation data because the termination of the logarithmic phase of growth was usually followed by a rapid drop in the dry weight of mycelium as a result of autolysis. In a continuous culture with sucrose limitation, the yield constant remained unchanged in the range of dilution rates from 0.08 to 0.15 hr^{-1} and equalled 0.43; this value decreased at dilution rates lower than 0.08 hr^{-1} or higher than 0.15 hr^{-1} to 0.32 (figure 4). The shape of the curve showing the relationship between the values of the yield constant and the dilution rate was the same for both sucrose concentrations used. A similar shape is usual in nonfilamentous organisms. Herbert *et al.* (1956) and Maxon and Johnson (1953) explain the decrease of the yield constant at low dilution rates by presuming that, thanks to endogenous respiration, more carbon from the carbon source is incorporated into carbon dioxide than into the cell matter. The decrease of the yield constant at high dilution rates is explained by the production of different compounds which are not formed at low dilution rates (Maxon and Johnson, 1953; Pirt, 1957).

Saturation constant (K_s). The relationship between the growth rate and the concentration of the limiting factor (Monod, 1950)

$$\mu = \mu_{max} \cdot \frac{s}{K_s + s}$$

certainly is too simple to express accurately the complicated process of growth (recently other, more complicated relationships have been postulated) but is quite helpful for such purposes where it is not necessary to express directly the reaction kinetics. This constant was not calculated from batch data, as in batch cultivation the logarithmic phase ends too abruptly. It cannot be calculated from data obtained in continuous cultivation at low dilution rates, for the concentration of the limiting substrate is too low and lies beyond the accuracy limit of the assay method. We saw little purpose in calculating it from values obtained at high dilution rates, respective from the D_w value (Herbert *et al.*, 1956), as these values are probably already influenced by the "apparatus effect" (wall growth and imperfect mixing). It can be said with certainty, however, that, as with bacteria, the K_s is very low because the logarithmic phase terminates very abruptly in batch cultivation and, in continuous cultivation, a very steep decline in the dry weight of mycelium occurs as soon as D_w is exceeded.

Influence of Type of Limiting Substrate and Dilution Rate on Nitrogen Content in Dry Weight of Mycelium

The data concerning changes of the nitrogen content in the dry weight of mycelium are in accordance with similar studies performed on bacteria by Holme (1957) and Formal, Baron and Spilman (1956). When considering the decrease of nitrogen content in the dry weight of mycelium in nitrogen limitation, accompanying the lowering of the dilution rate, it must be emphasized that, in our experience, this phenomenon cannot be explained by the production of nitrogen-free compounds in the mycelium (*e.g.*, glycogen (Holme, 1957)); there is, simultaneously with the decrease of nitrogen content in the dry weight of mycelium, a drop in the dry weight of mycelium itself. Instead, it seems probable that nitrogenous compounds are produced and released to the medium; this possibility is corroborated by the observed increase in concentration of amino-nitrogen at low dilution rates in nitrogen limitation.

Production of Chlortetracycline and Pigments in Continuous Culture of S. aureofaciens

In previously published papers, the continuous biosynthesis of antibiotics was aimed at the practical utilization of the continuous method. We have therefore in our experiments followed the production of an antibiotic under well-defined conditions in dependence on the dilution rate, and type and concentration of the limiting factor, even though the synthetic medium was not favourable to the antibiotic production. The concentrations of the antibiotic obtained are in fact very low, yet still exactly measurable.

From the above results, the sudden disappearance of the antibiotic and pigments at low dilution rates in sucrose limitation, and the gradual disappearance of the antibiotic and pigments in nitrogen limitation, are the most interesting. A similar observation was described by Bartlett and Gerhardt (1959) during the continuous biosynthesis of chloramphenicol (although, in their case, no substrate limitation was concerned), namely a decrease of the antibiotic content in the myce-

from resulting from a decrease of the dilution rate. The phenomenon seems therefore to be of a more general nature. Incidentally, it has been pointed out that even in the biosynthesis of simple products such products as are produced at high dilution rates often cease to be produced at low dilution rates (Mavon and Johnson, 1951; Pirt, 1957).

In our experience, two possible explanations present themselves for the loss of the antibiotic and pigment producing ability. At low dilution rates, when the concentration of the substrate in the cultivation vessel is low, the enzyme surfaces related to the production of the antibiotic and pigments are not saturated, so that neither the antibiotic nor the pigments are produced. Another explanation may be found in the selecting of a certain part of the heterogeneous population. A non-pigmented part of population, producing no antibiotic, might have a higher growth rate at low concentrations of the limiting factor (at low dilution rates), whereas a pigmenting and antibiotic-producing part of population might have a higher growth rate at a higher concentration of the limiting substrate (at a higher dilution rate). The second alternative is supported by the fact that the antibiotic and pigment production is not resumed immediately upon increasing the substrate concentration in the cultivation vessel. Undoubtedly, other explanations could be found, *e.g.*, the difference in the "physiological state" of the culture at different dilution rates, as stressed by Málek (1958). To solve this question and to explain the differences between nitrogen and sucrose limitation, further research is necessary.

Acknowledgment

The authors wish to thank V. Brynda and V. Kratochvíl for technical assistance.

Summary

The behaviour of a strain of *Streptomyces aureofaciens*, as representative of filamentous microorganisms, was studied during continuous growth in synthetic medium. It was found that under substrate limitation the behaviour of filamentous microorganisms is the same as nonfilamentous microorganisms. In both cases, the conformity with the theory is only fair and the discrepancies from the theory are of the same character. The production of chlortetracycline and pigment formation were also studied with regard to dependence on the type and concentration of the limiting substrate and the dilution rate.

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Journal of Biochemical and Microbiological Technology and Engineering
VOL. I, NO. 4, PAGES 379–390, 1959

Continuous Streptomycin Fermentation

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Summary. A pilot plant unit for continuous cultivation of hyphae-forming micro-organisms, providing for a uniform flow velocity in fermentations on suspension media with a massive growth of mycelium, is described. For the maintenance of aseptic conditions, over-pressure of air is maintained in the whole equipment. This equipment has been used for studies of the continuous biosynthesis of streptomycin. A three-stage fermentation proved the most advantageous. The first stage serves for multiplication of the inoculum; the second and third for the formation of the antibiotic. The system was maintained for 300–400 h with yields amounting to 2,000–2,500 u. of streptomycin /ml without any signs of contamination or degeneration of the growing culture.

Introduction

Continuous cultivation of micro-organisms is gaining ever-increasing importance in microbiology for the solution of both theoretical and practical problems, due to its numerous advantages compared with batch cultivation, as was pointed out by Novick.[1]

The basic theory of continuous cultivation was first published by Monod,[2] and Novick and Szilard,[3, 4] and further developed by numerous authors.[5–9] There exist two basic types of continuous cultivation method and two basic types of cultivation equipment. One of these is called 'chemostat',[3] or 'bactogen',[2] the other 'turbidostat'.[10] In the first method fresh medium is added at constant velocity to the cultivation vessel in which a constant volume of medium is maintained. The velocity of growth of the culture is limited by lowering the concentration of one of the basic nutrients, the other nutrients being in excess. In the second method, the equilibrium state is reached by increased velocity of inflow for an increasing number of micro-organisms in the vessel

and by decreased inflow for a decreasing number of micro-organisms in the vessel. The other types of equipment which have been described so far are more or less modifications of the two basic systems, e.g. the auxanometer,[11] the breeder,[12] and a number of others.[5, 13, 15]

The method of continuous cultivation of micro organisms has already been used in a number of varied applications: for the multiplication of a large number of pathogenic micro-organisms,[16, 17] in the field of microbial genetics in a study of mutation conditions,[4, 18] for the selection of bacterial cultures,[10] and for the study of enzyme formation.[2, 19, 20]

Simultaneously with the solution of theoretical questions, possibilities of application of the continuous method in various branches of the fermentation industry were considered. One of the very first publications on continuous cultivation[21] was concerned with the possibility of continuous production of lactic acid. Continuous propagation of baker's yeast,[22] of fodder yeast,[23, 24] of alcohol,[25, 26] and of butanol[27] were also objects of investigation. In the construction of equipment for continuous cultivation special emphasis has been laid upon the maintenance of a uniform flow of medium. Various devices for this purpose have been described: the principle of the Mariotte bottle,[13, 15, 28] rotameters[8, 29] and various types of pumps.[6, 30] In all the types of apparatus described so far, however, only clear, non-suspension type media may be used.

In industrial-scale production of antibiotics rich suspension-type media are used almost exclusively in order to secure maximum yields. One of the basic conditions of successful production of antibiotics is the maintenance of strictly aseptic cultivation conditions, achieved by an over-pressure of air in the whole equipment; this constituting a substantial difference from the production of baker's yeast, fodder yeast, and alcohol. A further factor, influencing profoundly the production of antibiotics, is aeration and agitation of the fermented liquid. During intense aeration, especially of suspension media, strong foam formation often takes place and defoaming is very difficult.

In this paper we describe an apparatus for the continuous cultivation of hyphae-forming micro-organisms, providing a uniform flow velocity even in fermentations on suspension media

with a powerful growth of mycelium. For aseptic cultivation conditions, air over-pressure is maintained in the whole equipment.

General Description

The equipment is shown schematically in Fig. 1. It consists of a tank (*S*) of 80 l. working capacity in which the medium is prepared and sterilized. From this tank, sterile medium is let

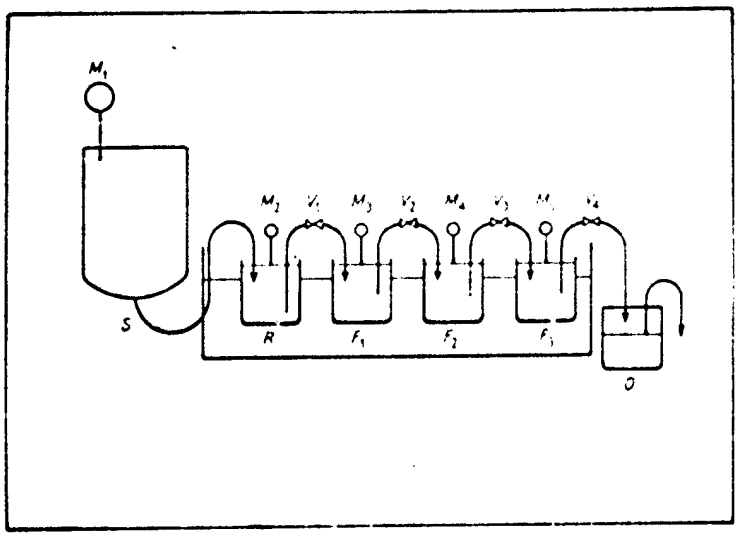

Fig. 1. Continuous culture plant

S sterilizing vessel
R reservoir
*F*1, *F*2, *F*3 fermentors
O collector
*V*1, *V*2 electromagnetic valves
*M*1 *M*5 manometers

out into a small tank (*R*) of 20 l. capacity, serving as an intermediate reservoir for medium while it is being prepared and sterilized in the large tank. From the small tank medium flows into one, two or three little tanks (*F*1, *F*2, *F*3) mutually connected, of 5–15 l. capacity, in which the cultivation proper takes place, in one, two or three stages. The fermented medium is collected in the collector (*O*).

The velocity of flow of medium is regulated by electromagnetic

valves (V_3–V_4). The valves are governed by two adjustable relay switches, one of which determines the opening time of the valve, the other the frequency of medium injections. For aseptic conditions of cultivation air over pressure is maintained in the whole equipment and measured by manometers (M_1 M_5). Air over-pressure decreases from the value of 0·8 atm in tank S to 0·2 atm in the collector O. Gradual decrease of over pressure in the sequence of vessels is necessary for functioning of the valves and for the regulation of flow velocity. For the maintenance of an equilibrium state in the growing culture the outflow velocity has to be equal to the inflow velocity. This involves the following items of equipment: the cultivation vessel for the preparation and sterilization of the medium, the reservoir, the electromagnetic valves, the relay switch, piping and collector. A detailed description of all these parts follows.

Cultivation Vessel

As fermentation vessels we used standard laboratory fermentation tanks, used in the Antibiotics Research Institute for studies in the technology of antibiotics, which have been slightly modified for continuous work. The tanks are made of stainless steel AKVN, the vessel itself being a cylinder with a total capacity of 20 l. (working capacity 5–15 l.). These tanks have been described in detail elsewhere.[31]

Sterilization Tank

For sterilization and preparation of the medium a stainless steel fermentation tank of 90 l. working capacity was used. The contents of the tank are agitated by a paddle impeller on a shaft. The shaft is driven by a 1·5 h.p. motor by means of a belt pulley. The tank is further equipped with a ring sparger and a baffle, and equipment for sampling and for injection of auxiliary substances. Medium is sterilized and cooled by means of coils with either steam or cold water. Air entering the tank through the ring sparger is sterilized by a filter packed with glass wool. On the lower end of the tank there is the closing valve, connected with the reservoir by means of piping.

shown schematically in Fig. 2. It functions as follows: by connecting the net switch V, the time relay (TM 100s), adjustable from 2 to 100 sec, is brought into action. This relay determines the 'closed' period between two injections. After this period, the relay connects the d.c. 6 V circuit feeding the coil of the telephone relay (Tel. Bv.), and the electrolytic condenser (60 μF) with a parallel wire potentiometer of 3·2 kΩ by which the duration of the injection is determined. This potentiometer dissipates, more or less according to its position, the charge on the condenser feeding the coil of the telephone relay. By attraction of the core of the relay Tel. Bv. the feeding circuit of the timing relay TM 100s is instantly interrupted, whereas the condenser continues to feed the coil of the relay Tel. Bv. until discharge. After that the core of the telephone relay falls again connecting the circuit of relay TM 100s determining again the interval between the injections. The relay RP 90 connects the circuit of the valves.

Maintenance of Constant Level of Medium in Fermentation Tanks

A constant volume of medium in the fermentation tanks is maintained by an overflow pipe reaching to the level of the fermentation liquid. The amount of outflowing liquid is governed by intermittent make-and-break of the electromagnetic solenoid valve, the function of which is synchronized with that of the valve for the inlet of medium.

Description of the Procedure

The sterilization tank is sterilized by steam passing through the coils in the tank. The reservoir, the fermentation tanks and the collector are independently sterilized in the box autoclave for 4 h at 125°C. Each tank is sterilized with the appropriate part of the piping and the closing valve without the electric coil, cover and cap. During sterilization all valves and taps of the tank are closed and free connections are wrapped in cotton wool. After sterilization the tanks are placed in the water bath. This is equipped with an automatic supply of cold water governed by a pneumatic registrating temperature regulator with a capillary thermometer as a sensing device and a pneumatic membrane valve. The reservoir (*R*) is connected with the sterilization

tank (*S*) by means of piping. The rest of the tanks (F_1-F_3) are so connected that the piping with the valve of one tank is connected with the corresponding part of the piping of the second tank by means of a cap nut. After the whole system has been so connected, the electric coils, covers and caps are fixed in place.

The medium is prepared and sterilized in the sterilization tank. After sterilization, medium from this tank is let into the other tanks except the collector. After consumption of all of the medium from the sterilization tank, this tank is used again for the preparation of new sterile medium. Medium in the tanks is inoculated with an inoculum from a flask by a pipette, supply and outlet of air is connected, and the aeration and over-pressure of air in the tank is regulated to the desired value.

Experimental Results

The equipment described above was used to study the possibility of continuous biosynthesis of streptomycin. *Streptomyces griseus*, strain LS–1, and medium of the following composition were used in the experiments: glucose 3·3 per cent, corn-steep solids 0·66 per cent, ammonium sulphate 0·9 per cent, monopotassium phosphate 0·02 per cent, calcium carbonate 0·6 per cent and ferrous sulphate 0·003 per cent.

The medium was aerated with 1·0 volume of air/min and stirred at 400 rev/min. The rate of oxygen transfer as measured by the sulphite oxidation was 2,500 ml O_2/l.h. The temperature during growth was maintained at 28°C and the tanks were inoculated with 100 ml of a 48 h inoculum from a flask.

Single-stage Process

At first, continuous cultivation in a single vessel was employed. Fig. 3 shows the patterns of pH and streptomycin concentration at a dilution rate of $D = 0{\cdot}02\ h^{-1}$. After starting the flow of the nutrient medium, the quantity of antibiotic constantly decreased and steadied at about 200 units of streptomycin/ml; when the flow of the nutrient medium was stopped, the quantity of antibiotic increased again until all glucose was utilized. Maintenance of a constant production of streptomycin between the

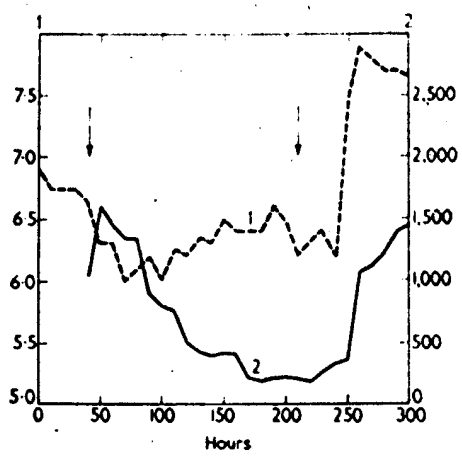

Fig. 3. Continuous cultivation of *Streptomyces griseus* at the dilution rate $D = 0{\cdot}02\ h^{-1}$. (1) pH; (2) concentration of streptomycin (u./ml). The arrows indicate the start and the end of the flow of the nutrient medium

limits of 2,000–2,500 u./ml was possible only with a very low dilution rate of $D = 0{\cdot}01\ h^{-1}$ (Fig. 4).

The process of biosynthesis for most antibiotics may be divided into at least two phases—a growth phase and a production phase.[32] The main synthesis of antibiotic does not begin until

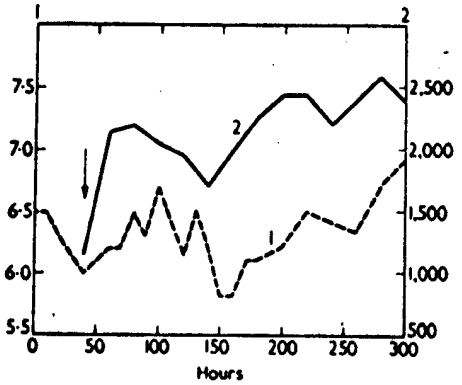

Fig. 4. Continuous cultivation of *Streptomyces griseus* at the dilution rate $D = 0{\cdot}011\ h^{-1}$. (1) pH; (2) concentration of streptomycin (u./ml). The arrow indicates the start of the flow of the nutrient medium

the growth of culture has largely stopped (Fig. 5). This is a typical example of a process in which the formation of the product is not connected with the growth of the micro-organism. The conversion of such a process into a one-stage, continuous process is therefore very difficult and may even be impossible because the dilution rate optimal for the growth of the mycelium is far greater than that for biosynthesis of antibiotic. We therefore considered the possibility of dividing the whole fermentation into several stages, with a three-stage process proving the most advantageous.

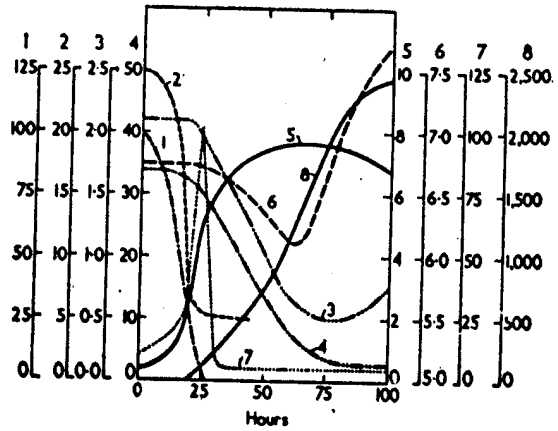

Fig. 5. Metabolic changes in the submerged culture of *Streptomyces griseus*. (1) concentration of phosphorus (μg/ml); (2) concentration of α-amino-nitrogen (mg/10 ml); (3) concentration of ammonia nitrogen (mg/ml); (4) concentration of glucose (mg/ml); (5) dry wt mycelium (mg/ml); (6) pH; (7) concentration of pyruvate (μg Na pyruvate/ml); (8) concentration of streptomycin (u./ml)

Multi-stage Operation

According to the course of metabolic changes determined in batch fermentation (Fig. 5) it is possible to divide the development of the culture of *Streptomyces griseus* into these three stages:

(1) Logarithmic growth of the mycelium accompanied by a rapid consumption of amino-nitrogen and phosphorus in the medium and by a steep increase and subsequent steep decrease of the level of keto-acids in the medium.

(2) Rapid consumption of reducing substances and ammonia

nitrogen, a decrease in pH and increasing formation of the antibiotic.

(3) Rapid synthesis of the antibiotic accompanied by a rapid increase in pH.

The operating process was divided into three steps corresponding to the above three stages; in the first stage continuous multiplication of the mycelium, in the second intense consumption of reducing substances and in the third main synthesis of the antibiotic, take place.

The most difficult problem was the determination of the velocity of exchange of one volume of medium in the first stage. From the curve of mycelial dry weight with time, determined for batch fermentations, the weight increase per hour between 10–60 h was calculated by a method described previously.[33] The shortest period of exchange of one volume of medium with maintenance of equilibrium conditions corresponded to a dilution rate of $D = 0{\cdot}2\ h^{-1}$; in this case, however, the amount of mycelium in the medium was low. A satisfactory concentration of the mycelium in the nutrient medium (about 3·5 mg dry weight/ml) was achieved at the dilution rate of $D = 0{\cdot}1\ h^{-1}$ (Fig. 6). As the

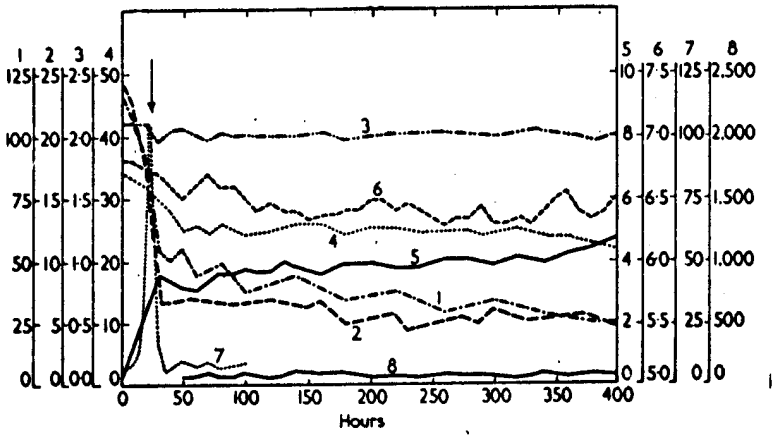

Fig. 6. Metabolic changes in the process of continuous cultivation of *Streptomyces griseus* in the first stage at the dilution rate $D = 0{\cdot}1\ h^{-1}$. (1) concentration of phosphorus (μg/ml); (2) concentration of α-amino-nitrogen (mg/10 ml); (3) concentration of ammonia nitrogen (mg/ml); (4) concentration of glucose (mg/ml); (5) dry wt mycelium (mg/ml); (6) pH; (7) concentration of pyruvate (μg Na pyruvate/ml); (8) concentration of streptomycin (u./ml). The arrow indicates the start of the flow of the nutrient medium

logarithmic growth of mycelium is accompanied by a parallel rapid increase of the level of keto-acids in the medium, flow was started when the maximum of keto-acids (about 100 μg/ml) was reached—usually about 23–24 h of cultivation.

Metabolic changes during the first, or rapid growth, stage of continuous fermentation are shown in Fig. 6. After the flow of the nutrient medium had been started (indicated by the arrow) a small decrease of the weight of mycelial dry matter set in and the weight did not stabilize at a constant value till after 50 h of cultivation. A similar decrease, occurring in the cultivation

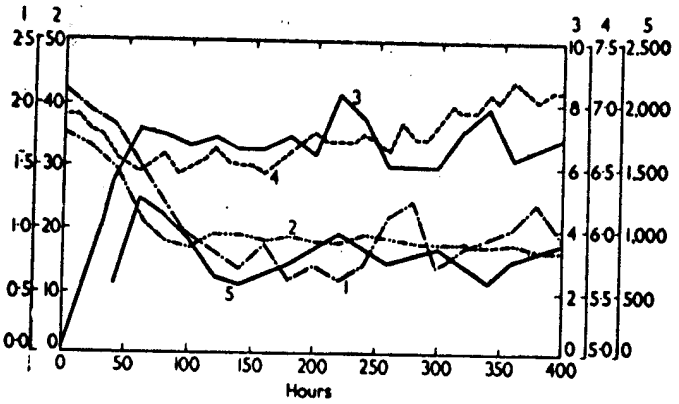

Fig. 7. Metabolic changes in the process of continuous cultivation of *Streptomyces griseus* in the second stage at the dilution rate $D = 0{\cdot}05\ h^{-1}$. (1) concentration of ammonia nitrogen (mg/ml); (2) concentration of glucose (mg/ml); (3) dry wt mycelium (mg/ml); (4) pH; (5) concentration of streptomycin (u./ml)

of bacteria, is ascribed by some workers to the distribution of generation times in the culture. It was found that the amount of ammonia nitrogen remained constant at the initial level during the whole process of fermentation while glucose and amino-nitrogen fell gradually. Investigations concerning the growth limiting factor were not successful. During the whole process of continuous fermentation it was possible to observe a small, gradual decrease in phosphorus and amino-nitrogen content and a slight increase in the weight of mycelial dry matter. This was most probably caused by the growth of micro-organisms on the walls of the fermenting vessel. These micro-organisms could be

washed down into the nutrient medium again, increasing the concentration of micro-organisms and causing an increased consumption of some of the nutrients. The amount of antibiotic in the medium varied from zero to 100 μ/ml in this first stage.

The process of the second stage is shown in Fig. 7. After 50 h of cultivation a strong growth of mycelium on the walls of the fermenting vessel was observed. The utilization of nutrients and the weight of mycelial dry matter varied considerably during the fermentation. Evidence was obtained that phosphorus and amino-nitrogen were entirely utilized so that they could be considered as factors limiting the growth. Adding both phosphorus and amino-nitrogen during fermentation did not, however, increase the weight of dry mycelium. Ammonia-nitrogen was partially utilized, but its content in the medium varied considerably in this stage. This may be explained by a partial autolysis of the hyphae which was also ascertained by microscopic observation. The content of streptomycin steadied after the initial increase within the limits of 500–1,000 u./ml.

It was very difficult to follow metabolic changes and the amount of mycelium in the medium during the third, final stage. The adherence of mycelium to the walls of the fermenting vessel

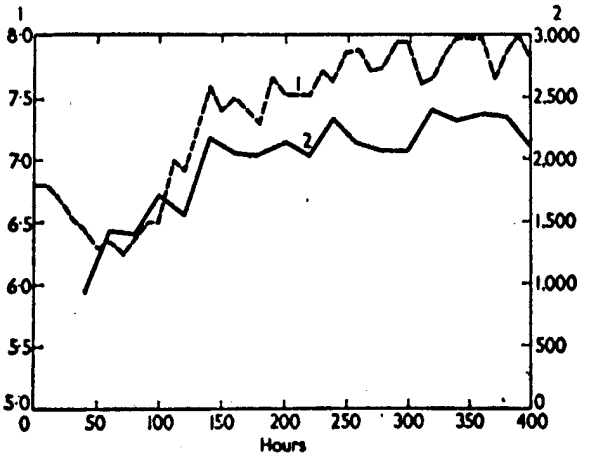

Fig. 8. Third stage continuous cultivation of *Streptomyces griseus* at the dilution rate $D = 0{\cdot}033\ h^{-1}$. (1) pH; (2) concentration of streptomycin (u./ml)

was very great and caused considerable differences in nutrient levels in the medium; therefore only the level of antibiotic and changes of pH were surveyed (Fig. 8). It is worth noting that even in this stage, at a low dilution rate, glucose and ammonia nitrogen were not entirely utilized and were removed unused. This factor, when applied to an industrial facility on a larger scale, would make this system less advantageous economically than the more common batch process.

In the whole equipment the flow velocity was so adjusted as to exchange the 5 l. of fermentation liquid in the first stage in 10 h. In the second stage a doubled residence time was achieved by doubling the volume of the liquid to 10 l. at equal flow velocity. In the third stage the volume was adjusted to 15 l. of medium for the same reason, giving a dilution rate of $D = 0{\cdot}033\ h^{-1}$.

The total fermentation operating time amounted to 300–400 h, with yields of 2,000–2,500 u. of streptomycin/ml, without any sign of degeneration or contamination of the culture.

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